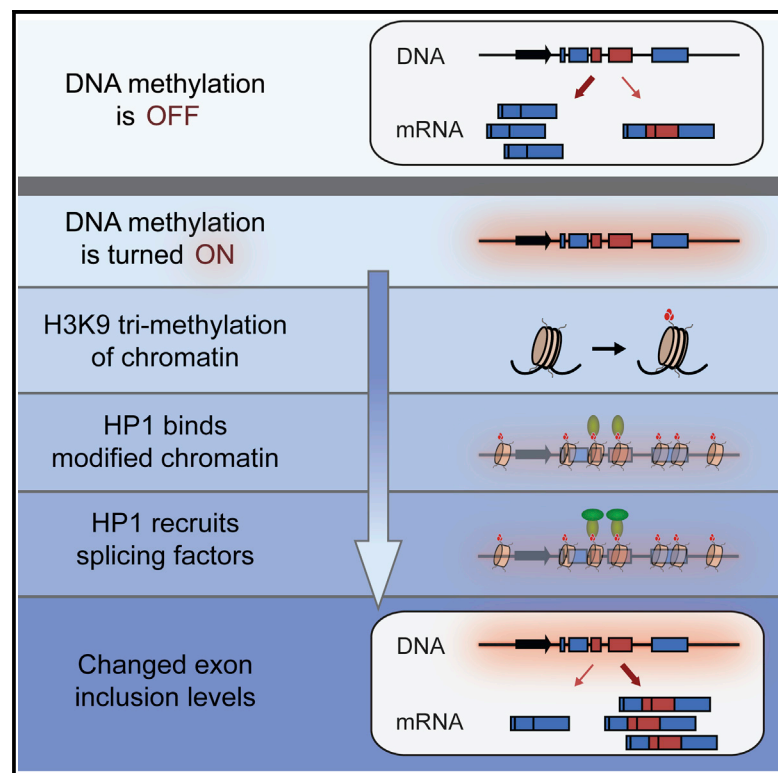


HP1 Is Involved in Regulating the Global Impact of DNA Methylation on Alternative Splicing

Graphical Abstract



Authors

Ahuvi Yearim, Sahar Gelfman, ..., Eran Meshorer, Gil Ast

Correspondence

meshorer@huji.ac.il (E.M.), gilast@post.tau.ac.il (G.A.)

In Brief

The global impact of DNA methylation on mRNA splicing is largely unknown. Yearim et al. characterize the genome-wide effect of DNA methylation on alternative splicing and demonstrate a direct relationship between DNA methylation and splicing. Additionally, they describe an adaptor system for splicing factor recruitment by DNA methylation through HP1 proteins.

Highlights

- DNA methylation directly affects mRNA alternative splicing
- Methylation-affected exons display distinct genetic and epigenetic signatures
- HP1 mediates DNA methylation's effect on splicing by recruiting splicing factors
- HP1 enhances or silences inclusion depending on its relative binding position

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HP1 Is Involved in Regulating the Global Impact of DNA Methylation on Alternative Splicing

Ahuvi Yearim,^{1,5} Sahar Gelfman,^{1,4,5} Ronna Shayevitch,¹ Shai Melcer,² Ohad Glaich,¹ Jan-Philipp Mallm,³ Malka Nissim-Rafinia,² Ayelet-Hashahar S. Cohen,² Karsten Rippe,³ Eran Meshorer,^{2,6,*} and Gil Ast^{1,6,*}

¹Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel-Aviv University, Ramat Aviv 69978, Israel

²Department of Genetics, Institute of Life Sciences, The Hebrew University of Jerusalem, Edmond J. Safra (Givat Ram) Campus, Jerusalem 91904, Israel

³Deutsches Krebsforschungszentrum (DKFZ) and BioQuant, Research Group Genome Organization & Function, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

⁴Center for Human Genome Variation, Duke University School of Medicine, Durham, NC 27708, USA

⁵Co-first author

⁶Co-senior author

*Correspondence: meshorer@huji.ac.il (E.M.), gilast@post.tau.ac.il (G.A.)

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SUMMARY

The global impact of DNA methylation on alternative splicing is largely unknown. Using a genome-wide approach in wild-type and methylation-deficient embryonic stem cells, we found that DNA methylation can either enhance or silence exon recognition and affects the splicing of more than 20% of alternative exons. These exons are characterized by distinct genetic and epigenetic signatures. Alternative splicing regulation of a subset of these exons can be explained by heterochromatin protein 1 (HP1), which silences or enhances exon recognition in a position-dependent manner. We constructed an experimental system using site-specific targeting of a methylated/unmethylated gene and demonstrate a direct causal relationship between DNA methylation and alternative splicing. HP1 regulates this gene's alternative splicing in a methylation-dependent manner by recruiting splicing factors to its methylated form. Our results demonstrate DNA methylation's significant global influence on mRNA splicing and identify a specific mechanism of splicing regulation mediated by HP1.

INTRODUCTION

DNA methylation is an important epigenetic mark, significantly contributing to natural human variation (Heyn et al., 2013). Examination of the full DNA methylome revealed that DNA methylation has dual and opposing roles in the regulation of gene expression. In promoter regions, DNA methylation is associated with transcriptional repression, while in gene bodies, DNA methylation is generally associated with high expression levels (Ball et al., 2009; Laurent et al., 2010; Rauch et al., 2009). The understanding that DNA methylation is significantly present in the bodies of

highly transcribed genes led to speculation about its possible biological role in transcription or subsequent processing of active genes.

Alternative splicing contributes to proteome diversity. At least 95% of human multi-exon genes produce alternatively spliced transcripts (Pan et al., 2008; Wang et al., 2008). High-resolution bisulfite sequencing of the genomes of several organisms showed an enrichment of DNA methylation in exons compared to introns (Chodavarapu et al., 2010; Hodges et al., 2009; Lyko et al., 2010). In addition, we previously found that gene regions encoding constitutively spliced exons display higher levels of methylation than those encoding alternatively spliced exons (Gelfman et al., 2013). DNA methylation was also recently found to be positively correlated with inclusion levels of alternative exons (Maunakea et al., 2013). These correlations prompted speculation that DNA methylation plays a role in the regulation of alternative splicing.

Given the fact that splicing occurs co-transcriptionally, there are two possible models for epigenetic regulation of splicing: (1) a kinetic model, in which an epigenetic modification affects the kinetics of transcriptional elongation that subsequently impacts splicing, and (2) a recruitment model, in which splicing regulation occurs through adaptor proteins that bind to epigenetic modifications and recruit splicing factors (Iannone and Valcárcel, 2013). Several recent studies now support methylation-regulated splicing via a kinetic model. Transcriptional repressors such as CTCF and MeCP2 were found to modify the elongation rate of RNA polymerase II (RNAPII) in a methylation-dependent manner, which in turn enhances (MeCP2) or disturbs (CTCF) the efficiency of splicing (Maunakea et al., 2013; Shukla et al., 2011). In support of the recruitment model for other epigenetic modifications, the histone modification H3K36me3 was found to recruit the splicing factors PTB (Luco et al., 2010) and SRSF1 (Pradeepa et al., 2012). However, to date, DNA methylation has not been found to participate in any recruitment mechanism that affects splicing.

For DNA methylation to regulate splicing, it likely has a mediator protein that can affect splicing regulation on the one hand

and is selective to DNA methylation on the other. One promising candidate is heterochromatin protein 1 (HP1), which has three isoforms in humans: HP1 α , HP1 β , and HP1 γ . All HP1 proteins bind directly via their chromodomains to H3K9me3 (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002), a histone modification that is induced by DNA methylation (Hashimshony et al., 2003). Some HP1 isoforms have been previously linked to splicing regulation in specific cases (Alló et al., 2009; Ameyar-Zazoua et al., 2012; Saint-André et al., 2011; Schor et al., 2013; Smallwood et al., 2012). The combined evidence that HP1 can act as a splicing regulator in some contexts and also associates with H3K9me3, a histone modification that is co-localized with regions of methylated DNA, makes HP1 a potential candidate protein that regulates alternative splicing in a methylation-dependent manner.

Given that DNA methylation plays a role in the regulation of mRNA splicing, a whole-genome analysis can decipher the extent and magnitude of the global impact of DNA methylation on splicing and uncover underlying trends and patterns. To what extent does DNA methylation affect splicing? Does it affect all exons or only a specific group with specific characteristics? Also, do HP1 proteins work in concert with methylation to create a combined regulatory effect? To address these questions and more, we used a genome-wide approach and found that more than 20% of all alternative exons are affected by the absence of DNA methylation; for these exons, DNA methylation acts as either an enhancer or a silencer of splicing recognition. We characterized these methylation-affected exons and found that they possess specific genetic and epigenetic signatures that distinguish them from other exons. We applied our genome-wide approach to analyze the effect of HP1 proteins on splicing and found that these proteins also affect the inclusion level of a large amount of alternative exons, again acting as either splicing enhancers or silencers depending on the context of their binding to the exon. Splicing regulation through HP1 explains a significant portion of the overall effect of DNA methylation on splicing. To validate these findings, we established a novel experimental system that specifically targets DNA methylation to a single gene, and to that gene alone, and offer the first clear evidence that DNA methylation regulates alternative splicing directly, acting as a splicing enhancer in this case. HP1 also regulates alternative splicing in this system and acts as a splicing silencer. Finally, we decipher HP1's regulatory mechanism and provide a strong link between DNA methylation and alternative splicing via a recruitment model, i.e., by recruiting splicing factors to methylated alternative exons through an adaptor protein, HP1.

RESULTS

Altered DNA Methylation Patterns Affect Alternative Splicing Globally

The emerging picture from previous studies supports an important link between DNA methylation and splicing (Gelfman et al., 2013; Maunakea et al., 2013; Shukla et al., 2011). However, the extent and magnitude of this impact and its underlying trends and patterns are still not fully known. To address this issue, we performed RNA sequencing (RNA-seq) experiments on wild-type (R1) and Dnmt1/3a/3b triple-knockout (TKO) mouse embry-

onic stem cells (ESCs) that lack DNA methyltransferase activity, which results in unmethylated DNA in these cells (Melcer et al., 2012; Tsumura et al., 2006). The TKO cells continue to grow robustly, maintain their undifferentiated characteristics, and display morphological features similar to wild-type undifferentiated ESCs (Tsumura et al., 2006). We determined the inclusion levels of 14,987 alternative cassette exons in each cell type using RNA-seq. We next filtered the results based on very stringent read-depth criteria (see Supplemental Experimental Procedures). Of 3,376 alternative exons that met our criteria, inclusion levels of 752 (22.2%) differed by more than 10% in TKO compared to wild-type cells: 271 exons (8% of all alternative exons) decreased their inclusion level in TKO cells, which means that they were positively affected by methylation in the wild-type cells, and 481 exons (14.2% of all alternative exons) increased their inclusion level in TKO cells and thus were negatively affected by methylation in the wild-type (Figures 1A and S1A). These results indicate that DNA methylation can influence alternative splicing in both directions, having both positive (enhancing) and negative (silencing) roles in exon recognition. RT-PCR analysis of selected exons further validated these findings, supporting RNA-seq results in 93% of the cases (Figure S1B). We analyzed RNA expression levels of all spliceosome components in wild-type and TKO cells and found no significant changes (Table S1), implying that most of the changes in alternative splicing in TKO cells are a direct outcome of DNA methylation removal and not variations in quantities of splicing factors. We also performed differential expression analyses between wild-type and TKO cells for all mouse genes, revealing only minor overall differences in expression (Table S2). This finding fits well with previous knowledge that in mouse ESCs, CpG island promoters are not usually repressed by DNA methylation and are instead silenced by H3K27 methylation (Smith and Meissner, 2013).

To study the connection between a methylation-related effect and DNA methylation signal patterns, we performed whole-genome bisulfite sequencing at 10 \times coverage in wild-type R1 cells and mapped the methylation profile at single-base resolution. We then examined methylation levels in exons and their flanking intronic regions. Constitutive exons exhibited high methylation levels, whereas alternative exons, in general, exhibited lower levels of methylation (Figure 1B), as was also shown previously (Gelfman et al., 2013). This can imply that DNA methylation promotes exon inclusion, but our results suggest that the underlying mechanism is more complex. Constitutive exons in wild-type cells remain constitutive for the most part in TKO cells (98.5% of exons; data not shown), probably since their sequence-based splicing signals strongly support recognition by the splicing machinery, overshadowing the weaker DNA methylation effect. However, alternative exons that are more sensitive to regulation by DNA methylation show different levels of DNA methylation (Figure 1B). Exons that were negatively affected by methylation had significantly higher levels of methylation than positively affected exons (+40%–50% exonic; t test, p value < 3.24 \times 10⁻¹⁰), while alternative exons unaffected by methylation were found exactly between these two groups, with intermediate methylation values. This result suggests that when the exonic splicing signals are weaker (as is the case with alternative exons)

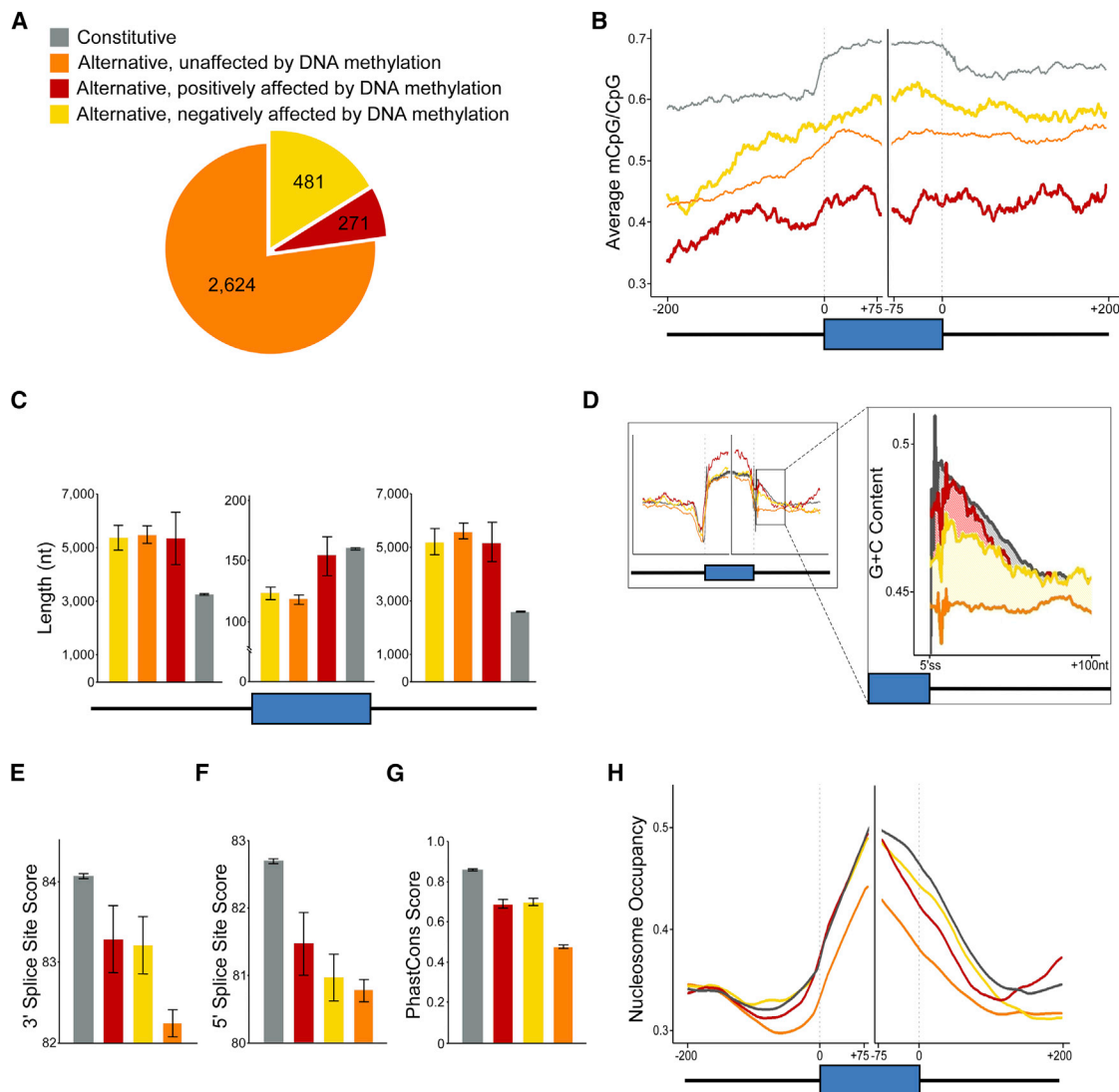


Figure 1. The Global Effect of DNA Methylation on Splicing Regulation

(A) Distribution of alternative exons based on direction of change in splicing pattern from TKO (unmethylated) to R1 (methylated) cells.

(B) Average of methylated CpGs for alternative (three groups) and constitutive exons.

(C) Average length of upstream introns (left), exons (center), and downstream introns (right) for alternative (three groups) and constitutive exons.

(D) G+C content for alternative (three groups) and constitutive exons. Right panel is a close-up of the first 100 nt downstream of the 5' splice site (5'ss).

(E) Average score of the 3' splice sites for alternative (three groups) and constitutive exons based on a PSSM calculation (Amit et al., 2012).

(F) Average score of the 5' splice sites for alternative (three groups) and constitutive exons.

(G) PhastCons conservation values for alternative (three groups) and constitutive exons. Error bars represent the SEM.

(H) Nucleosome occupancy in R1 cells for alternative (three groups) and constitutive exons.

The average values given in (B), (D), and (H) were calculated per base for exons (75 nt from each splice site) and flanking intronic regions (200 nt), and a running average was applied. See also Figure S1.

and the recognition of the exon is not strongly controlled by the basic splicing recognition factors, DNA methylation allows the fine-tuning of this recognition. In this case, high methylation levels will repress recognition of exons, while low methylation levels will enhance their recognition.

We next sought to better understand what defines the subgroups of exons that are regulated by DNA methylation. Interestingly, exons affected by methylation, especially those with higher

levels of inclusion in wild-type cells, show a resemblance to constitutively spliced exons in several attributes. Usually, the average length of alternatively spliced exons is relatively short compared to constitutive exons (Gelfman et al., 2012). However, exons that are included in a methylation-dependent manner are longer, with an average length comparable to constitutive exons (Figure 1C). Moreover, similarly to constitutive exons, methylation-dependent exons have a high G+C content

downstream of the 5' splice site, whereas exons unaffected by methylation do not (Figure 1D). This downstream G+C signal was previously shown to have a splicing regulatory role (Amit et al., 2012). Last, all methylation-affected exons possess stronger 3' splice sites than those unaffected by methylation, again more in resemblance to constitutive exons (Figure 1E). This difference in splice site strength was found mainly in 3' splice sites and was not pronounced for 5' splice sites, suggesting that it is not a result of G+C structure at the splice site (Figure 1F). This overall resemblance places exons that are regulated by DNA methylation on the scale of several factors somewhere in between unaffected alternative exons and constitutively spliced exons. Overall, alternative exons whose recognition is sensitive to DNA methylation appear to possess particularly strong factors for recognition yet are not strong enough to overcome the fine-tuned regulation by methylation (as is the case with constitutive exons).

We investigated evolutionary conservation of the different exons that are either regulated or unaffected by DNA methylation (see Supplemental Experimental Procedures). Interestingly, we found that methylation-affected exons are significantly more conserved than methylation unaffected exons (Figure 1G; Mann-Whitney test, p value $< 2.2 \times 10^{-16}$ and p value $= 2.8 \times 10^{-11}$ for negatively and positively affected exons, respectively). This finding highlights the biological importance of alternative exons that are regulated by DNA methylation, as they appear to be under higher selective pressure compared to other alternative exons.

We and others previously observed a correlation between nucleosome occupancy and exon recognition (Schwartz et al., 2009; Tilgner et al., 2009), and DNA methylation was recently shown to regulate nucleosome occupancy (Huff and Zilberman, 2014). To examine the possible role of nucleosome occupancy levels in methylation-dependent exons, we performed MNase-sequencing experiments to map nucleosome occupancy in the various exon groups. Alternative exons unaffected by methylation displayed a weaker nucleosome signal than did constitutive exons, as was previously observed for alternative exons in general (Schwartz et al., 2009; Tilgner et al., 2009). However, methylation-dependent alternative exons displayed levels of nucleosome occupancy similar to those of constitutive exons in both wild-type and TKO cells (Figures 1H and S1C, respectively). This result supports the previous claim, which places exons that are regulated by DNA methylation somewhere in between unaffected skipped exons and the highly recognized constitutively spliced exons.

Since DNA methylation can modulate RNAPII kinetics (Maunakea et al., 2013; Shukla et al., 2011), we asked whether this is also the case for our methylation-affected exons. We examined RNAPII occupancy using publicly available chromatin immunoprecipitation sequencing (ChIP-seq) data from mouse ESCs (Tiwari et al., 2012). The results exhibit a strong accumulation of RNAPII immediately upstream of positively affected exons, while no such accumulation was found on other exons (Figure S1D). This result suggests that RNAPII pauses near exons that are positively affected by methylation, pointing to RNAPII pausing as a possible mechanism for the positive effect of DNA methylation on splicing, supporting other mechanisms of

action through the kinetic model such as the case with CTCF (Shukla et al., 2011) and MeCP2 (Maunakea et al., 2013).

HP1 β and DNA Methylation Work in Concert to Regulate Splicing Decisions

To this point, we have shown that DNA methylation's effect on splicing can be bi-directional—it can enhance or suppress inclusion level of alternative exons. Previous studies have uncovered two mechanisms for methylation-dependent regulation of splicing (Maunakea et al., 2013; Shukla et al., 2011). However, these two mechanisms combined can only explain a small fraction of the overall regulatory effect of DNA methylation on splicing. Our search for other candidates that can directly affect splicing through DNA methylation has led us to HP1 proteins. As detailed above, previous evidence links HP1 to splicing regulation in some specific contexts, while other evidence links HP1 to H3K9me3, a heterochromatin-associated histone modification that can be co-localized with regions of methylated DNA. Combining this evidence makes HP1 a promising candidate to connect DNA methylation and splicing. To extend our observations on DNA methylation and to test the contribution of HP1 proteins on splicing in a genome-wide manner, we performed RNA-seq experiments on wild-type mouse ESCs, after knocking down each and all three HP1 isoforms (Figure 2A). RNA-seq experiments showed that there is a strong overlap among the three HP1 isoforms in the groups of affected exons, which encompasses approximately 70%–80% of the affected exons (Figure S2). This means that exons that were affected in a specific manner (positive or negative) by one isoform were usually affected in the same manner by the other two isoforms. Interestingly, this result suggests that the function of regulating alternative splicing is not limited to any single HP1 isoform. In fact, all three HP1 proteins probably share similar modes of action for alternative splicing regulation. This strong overlap in effects of all HP1 isoforms enabled us to take HP1 β as a sole representative of this protein family and examine its effects on splicing. Of 2,837 alternative exons that met our strict criteria, 1,266 (44.6%) exons changed their inclusion levels by more than 10% when HP1 β was knocked down (Figure 2B). Of these, 950 (33.5%) decreased their inclusion level and thus were positively affected by HP1 β , and 316 (11.1%) increased their inclusion level and thus were negatively affected by HP1 β . These results indicate that HP1 β (as also HP1 α and HP1 γ), akin to DNA methylation itself, can influence alternative splicing in both directions, having both positive (enhancing) and negative (silencing) roles in exon recognition. We found that exons positively affected by HP1 β are very long, as are exons positively affected by DNA methylation, while negatively affected exons are short (Figure 2C).

To examine whether splicing regulation by DNA methylation is related to HP1's effect, we looked for the exons that are affected in the same manner (either positively or negatively) by both DNA methylation and HP1. The results present an overlap between exons affected similarly by DNA methylation and HP1 β that is highly significant compared to a random distribution (Figures 2D and 2E; proportions test, p value $< 2.2 \times 10^{-16}$; see Supplemental Experimental Procedures). This points to a strong correlation between the mechanisms of action involving DNA methylation and HP1 β , as 152 exons, corresponding to 20%

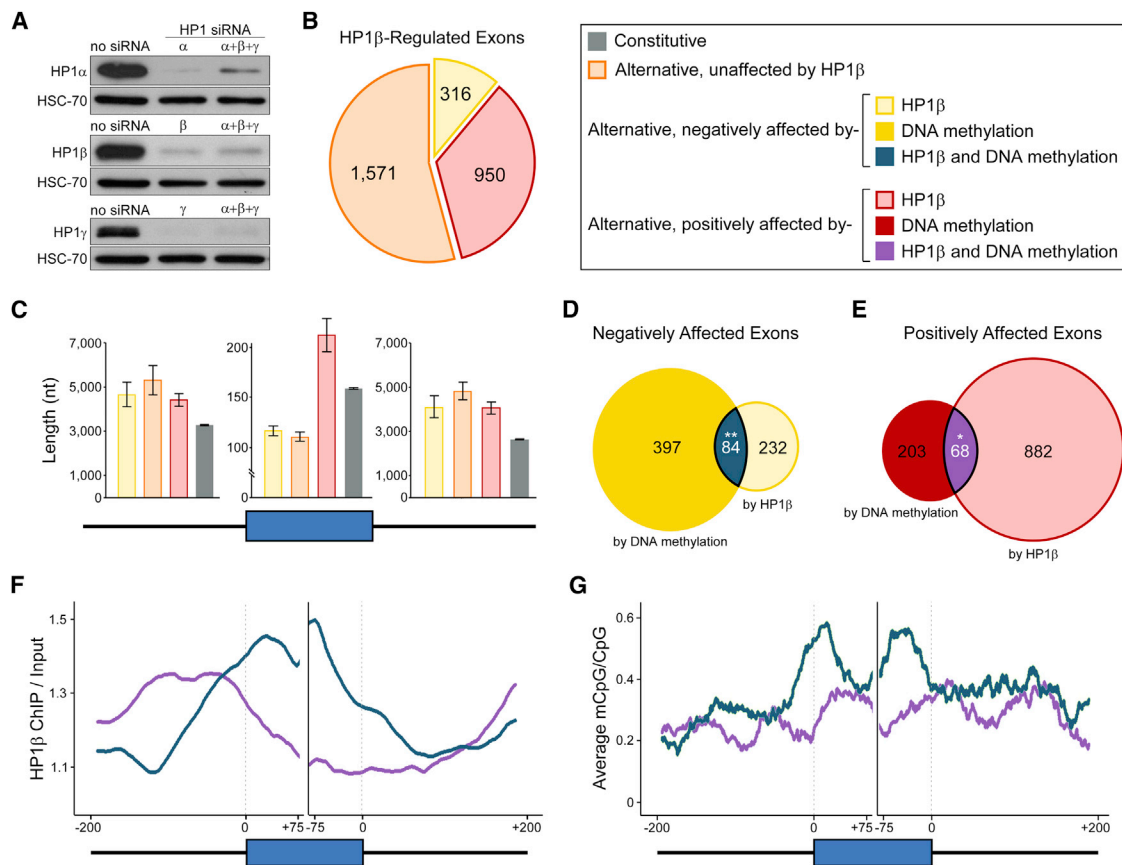


Figure 2. Identification and Characterization of HP1 β - and DNA Methylation-Dependent Exons

(A) Western blot analysis of protein extracts from R1 wild-type cells treated with indicated siRNAs. HSC-70 was used as loading control. (B) Distribution of alternative exons based on direction of change in splicing pattern between R1 cells treated with HP1 β siRNA and untreated cells. (C) Average length of upstream introns (left), exons (center), and downstream introns (right) for alternative (three groups) and constitutive exons. (D) Overlap of exons that were negatively affected by DNA methylation and HP1 β . (E) Overlap of exons that were positively affected by DNA methylation and HP1 β . (F) Normalized HP1 β ChIP-seq read signal of negatively (dark green) and positively (purple) affected overlapping exons based on (C) and (D). (G) Average of methylated CpGs of negatively (dark green) and positively (purple) affected overlapping exons. *Proportions test, $p < 1.3 \times 10^{-14}$; **proportions test, $p < 2.2 \times 10^{-16}$. See also Figure S2.

(152/752; Figures 2D and 2E) of the methylation-affected exons, can be explained by HP1 regulation. To study the biological function of the combined effects of DNA methylation and HP1, we performed Gene Ontology analysis using the DAVID tool (Huang da et al., 2009). Exons that are downregulated by both HP1 and methylation show strong enrichment for genes that take part in cell differentiation processes (p value = 0.005). This finding is of strong value, since the high methylation levels found in this group are a strong property of ESCs (Lister et al., 2009), where regulation of genes involved in differentiation is of major consequence. Indeed, when we attempt to differentiate the TKO cells by retinoic acid treatment, the cells die after 5 days of treatment, achieving only partial differentiation. This observation fits well, as misregulation of genes important for cell differentiation can lead to abnormal differentiation and subsequently to cell death. On the other hand, exons that are upregulated by both factors are enriched in genes that take part in translation regulation (p value = 0.002) and can play a role in

regulating gene expression in different tissues and multiple developmental stages.

To examine HP1 β binding profiles in the two exon groups (exons that are up- or downregulated by both HP1 and DNA methylation), we analyzed recently published HP1 β ChIP-seq data of wild-type mouse ESCs (Müller-Ott et al., 2014). Remarkably, alternative exons that were negatively regulated by both methylation and HP1 β showed a significant HP1 β signal within the exon itself (t test, p value $< 2.2 \times 10^{-16}$), whereas exons that were positively regulated by both methylation and HP1 β showed HP1 β enrichment in the upstream intron (Figure 2F). We also examined DNA methylation patterns on these exons. Consistently with the HP1 β signal, we found strong methylation signals at both ends of the negatively affected exons (Figure 2G), while the positive effect was consistently accompanied by low methylation levels (see Figure 1B). These results strongly suggest that HP1 β , a DNA binding protein, can affect RNA alternative splicing in a context-dependent manner: it acts as a splicing

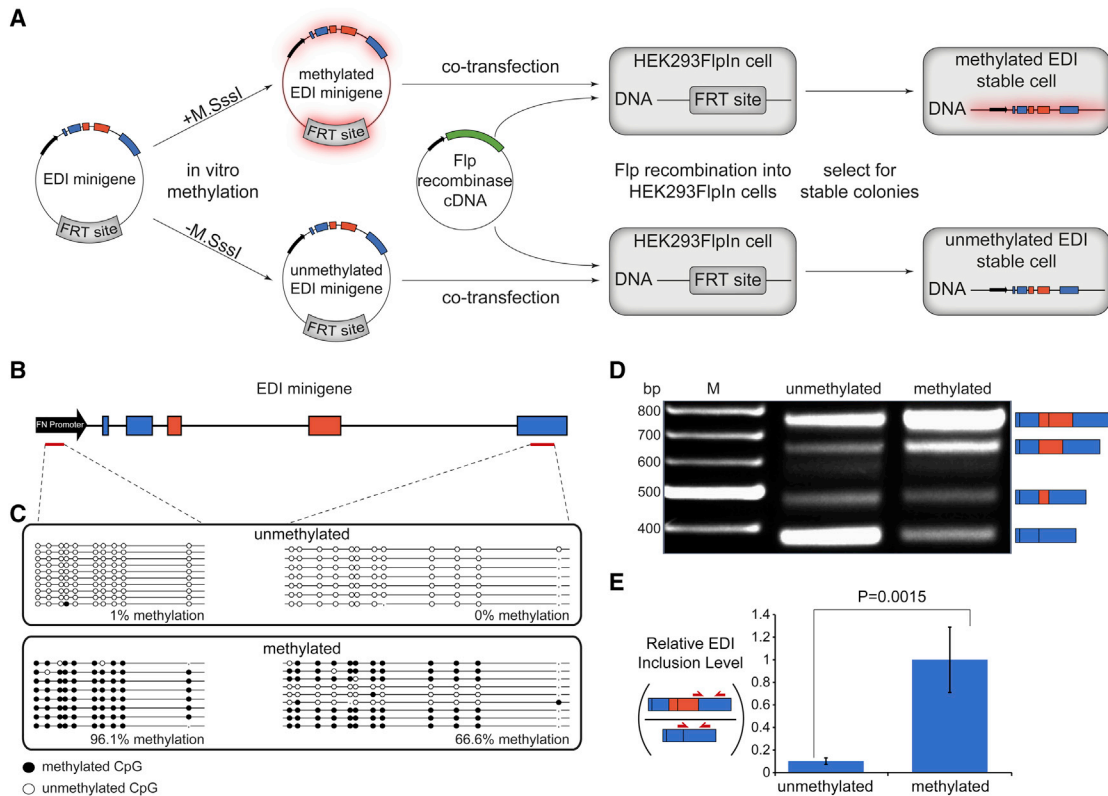


Figure 3. DNA Methylation Directly Affects Alternative Splicing

(A) Diagram illustrating the construction of the methylated and unmethylated EDI minigene experimental system.

(B) Schematic diagram of the EDI minigene. Constitutive and alternative exons are shown in blue and orange, respectively.

(C) Bisulfate sequencing of two representative regions (in red) on genomic DNA from cells containing unmethylated and methylated EDI. Below each plot is the methylation percentage of each region.

(D) RT-PCR of RNA from cells containing unmethylated and methylated EDI. The upper and lower bands represent inclusion and skipping of alternative exons, respectively.

(E) qRT-PCR of RNA from cells containing unmethylated and methylated EDI. Exon-exon junction primers for each isoform are represented by red arrows. EDI inclusion level was calculated by dividing amount of the inclusion isoform by amount of the skipping isoform. Values represent the mean \pm SEM (n = 4). Student's t test was used to compare the indicated samples. See also Figure S3.

silencer when bound to a methylated exon and as a splicing enhancer when it binds immediately upstream to long exons.

Targeted Methylation of a Single Gene Supports a Direct Methylation Effect on mRNA Splicing

Thus far, we have shown that DNA methylation can affect exon splicing bi-directionally. We next aimed to validate our genome-wide results in an isolated and carefully controlled system. To establish a causative connection between the factors at hand, we constructed an experimental system in which differential DNA methylation could be limited to a single gene while all other cellular factors remain identical. For that purpose, we constructed a novel biological system that takes advantage of the fibronectin EDI minigene. This minigene system offers a well-established model system that has been used extensively in the past to study splicing regulatory mechanisms (Iannone and Valcárcel, 2013). We introduced this minigene, either in vitro CpG methylated or unmethylated (see Supplemental Experimental Procedures), into human Flp-In-HEK293 cells using targeted Flp recombination (Figures 3A and 3B). This system offers three

advantages. First, the system enabled us to evaluate the effect of DNA methylation on one gene while the rest of the genome was unperturbed. Second, the gene was integrated into the genome and was thus studied in a normal genomic context. Third, methylated and unmethylated versions were introduced in the same orientation into the same genomic location, thus eliminating any confounding positional or other background effects. Following genomic insertion, we confirmed the methylation state using bisulfite sequencing for two representative regions and found that unmethylated EDI remained unmethylated, while methylated EDI remained mostly methylated after integration into the cells (Figure 3C). We next examined the splicing pattern of methylated versus unmethylated EDI exons using RT-PCR and qRT-PCR. Inclusion levels of the alternatively spliced exons were significantly higher in cells containing the methylated EDI gene than in cells containing the unmethylated EDI gene (Figures 3D and 3E). The endogenous EDI exon also displays a similar pattern, where high levels of DNA methylation coincide with high exon inclusion (Figure S3). Our genome-wide results indicate that DNA methylation can act as a splicing enhancer on

certain exons and as a splicing silencer on another fraction of exons (Figure 1A). The EDI minigene represents an example for a splicing enhancing ability. This effect is strongly supported by the genome-wide analysis (see Figure 1) when considering the similar structural features of the two EDI alternative exons and the generally affected exon population; both EDI exons display a high G+C content signal on the exon sequence and the sequence downstream of the 5' splice site. Additionally, the second alternative exon is very long (270 nt), and both exons have a stronger 3' splice site. Most importantly, this result provides for the first time a direct and causal link between DNA methylation and splicing. It indicates that DNA methylation, specifically on the EDI minigene, is directly involved in splicing regulation of its alternative exons. This result is accentuated by the fact that both cell types, harboring either a methylated or an unmethylated EDI minigene, share identical genomic and epigenomic backgrounds.

HP1 Mediates DNA Methylation and Splicing

Our genome-wide results indicate that HP1 proteins can regulate alternative splicing bi-directionally, explaining a substantial portion of DNA methylation's global effect on splicing. We next used our EDI experimental system to validate these genome-wide results regarding HP1's part in this mechanism. To test if HP1 serves as a mediator connecting DNA methylation with splicing, we first examined the role of HP1 proteins in our system by overexpressing or knocking down each of the three HP1 isoforms (Figure S4A). In the case of an unmethylated EDI, both overexpression and knockdown of all HP1 isoforms caused a general slight decrease in EDI inclusion levels (Figures S4B and S4C). In contrast, when EDI is methylated, HP1 manipulation gave a differential effect: overexpression of HP1 caused a prominent decrease in EDI inclusion (Figure S4B), while knockdown of HP1 caused an increase in EDI inclusion levels (Figure S4C). Given that the splicing effect on unmethylated EDI was similar for both overexpression and knockdown of all HP1 isoforms, we regard this as a background effect, which is probably indirect. To normalize this background, we divided the effect of changed HP1 levels on methylated EDI by the respective effect of changed HP1 levels on unmethylated EDI. Knockdown of HP1 α and HP1 β isoforms caused a stronger shift toward higher inclusion for a methylated EDI minigene, whereas overexpression of these isoforms produced the opposite effect (Figure 4A). Notably, changes in levels of HP1 γ did not significantly affect the methylation-induced shift in splicing patterns. These results suggest that HP1 α and HP1 β suppress EDI exon recognition in a methylation-dependent manner. Our genome-wide results indicate that HP1 can act as a splicing enhancer on a certain group of exons and as a splicing silencer for a different group of exons (Figure 2B). In this system, we observe an example for its ability to interfere with exon recognition when the exon is methylated. Here, we find that DNA methylation's "natural" effect is to enhance exon recognition. However, methylation-dependent binding of HP1 α and HP1 β to the minigene disrupts EDI exon recognition. DNA methylation can regulate splicing through multiple factors in addition to HP1, both known (Maunakea et al., 2013; Shukla et al., 2011) and unknown. Here, we observe a possible competition between factors, meaning that the

binding of other methyl binding regulatory proteins might exert a regulatory effect on EDI alternative splicing that is in competition with HP1.

To search for the mechanism by which this methylation-dependent regulation by HP1 occurs, we first investigated how HP1 proteins bind to the EDI gene using ChIP-qPCR. Importantly, we found significant enrichment of all three HP1 isoforms across the methylated, but not the unmethylated, EDI gene, indicating their expected association with methylated DNA. Furthermore, all HP1 proteins show specific binding peaks on the two methylated alternative exons and in the promoter region (Figure 4B). This result is fully consistent with our genome-wide observations, showing that HP1 silenced exon recognition when it bound to the alternative exon itself (Figure 2F).

It was previously shown that H3K9me3 functions as a substrate for HP1's binding to chromatin (Bannister et al., 2001; Jacobs and Khorasanizadeh, 2002). We therefore used ChIP-qPCR analysis to examine where along the minigene H3K9me3 is located. We found enrichment patterns similar to that of HP1, i.e., a strong enrichment on the methylated, but not the unmethylated, EDI gene, with specific peaks on the alternative exons and the promoter region (Figure 4B). These results show high correlation between HP1 isoforms and H3K9me3 along the minigene, specifically when alternative exons are affected. Comparing the signal of H3K9me3 to the nucleosome occupancy signal on the methylated EDI (Figure S4D; histone H3) allowed us to safely conclude that H3K9me3 is not a byproduct of increased nucleosome occupancy. Also, other histone marks such as H3K9me2 and H3K36me3 were largely unaffected by EDI methylation, supporting the expected result that indeed HP1 isoforms are associated via H3K9me3 (Figure S4D).

HP1 Recruits the Splicing Factor SRSF3

HP1 was previously shown to be associated with splicing factors SRSF1 in mitotic HeLa cells (Loomis et al., 2009), and heterogeneous nuclear ribonucleoprotein particles (hnRNPs) in *Drosophila* and humans (Ameyar-Zazoua et al., 2012; Piacentini et al., 2009). To examine whether this interaction occurs in our system, we performed co-immunoprecipitation assays to probe for the interaction of HP1 proteins with various splicing factors, which we suspected could associate with HP1. Of the examined splicing factors, we found that SF3B1, SRSF1, and SRSF3 bind to HP1 α and HP1 β isoforms, but not to HP1 γ (Figure 4C). This interaction was not sensitive to RNase treatment (see Experimental Procedures). We did not find HP1 to associate with other splicing factors such as SRSF6 and hnRNP A2/B1. We also did not detect any association between HP1 isoforms and RNAPII (Figure 4C), although this interaction was previously reported in *Drosophila* (Piacentini et al., 2009). Our finding that HP1 associates with SF3B1, a U2 small nuclear ribonucleoprotein particle (snRNP)-associated protein, led us to examine whether the whole U2 snRNP associates with HP1. Further co-immunoprecipitation experiments showed that all other tested U2 snRNP components also associated with HP1 (Figure S4E), which suggests that the whole U2 snRNP could be associated with HP1 and that HP1 might help recruit U2 snRNP to H3K9 methylated histones, similarly to CHD1's known recruitment of U2 snRNP to H3K4 methylated histones (Sims et al., 2007). Overall, the

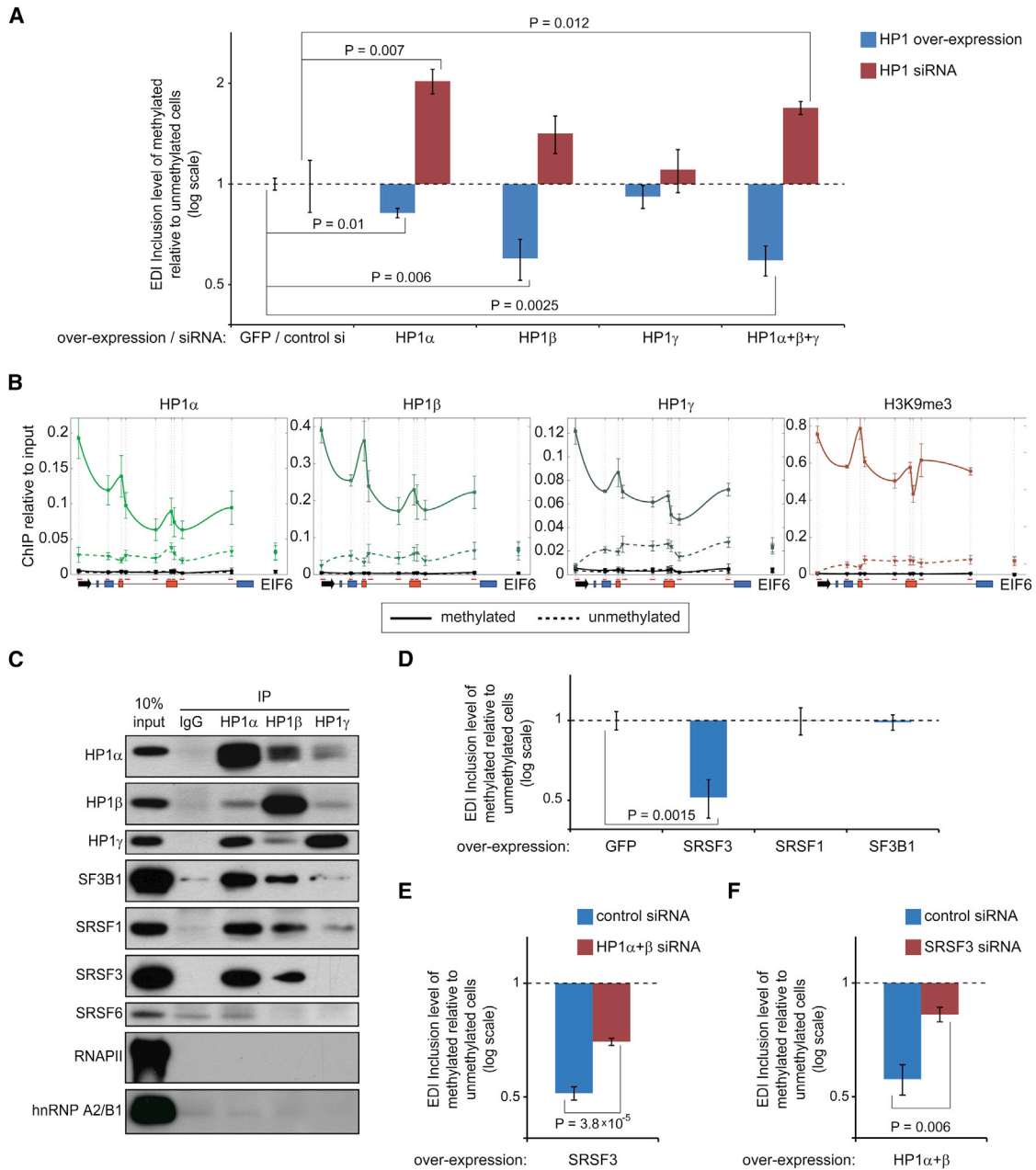


Figure 4. HP1 Mediates Splicing Changes in a Methylation-Dependent Manner by Recruiting SRSF3

(A) HP1 was overexpressed or knocked down using siRNA in methylated and unmethylated cells, and qRT-PCR analysis using the previously indicated exon-exon junction primers was used to assess EDI inclusion level of methylated relative to unmethylated cells (n = 3).

(B) ChIP of HP1 isoforms and H3K9me3 in unmethylated and methylated cells and qPCR along the EDI minigene using EIF6 intron 3 for endogenous background control. Values are ChIP relative to input (n = 3). Dotted and full lines represent the ChIP signal in unmethylated and methylated cells, respectively. Black lines represent ChIP with rabbit IgG.

(C) Co-immunoprecipitation of HP1 isoforms with various splicing factors and RNA polymerase II (RNAPII) in Flp-In-HEK293 cells.

(D) Indicated splicing factors were overexpressed, and qRT-PCR analysis was used to assess EDI inclusion level of methylated relative to unmethylated cells (n = 5).

(E) SRSF3 was overexpressed and cells were treated with HP1 siRNA (red) or control siRNA (blue; n = 5). EDI inclusion levels of methylated relative to unmethylated cells were determined.

(F) HP1 α and HP1 β were overexpressed, and cells were treated with SRSF3 siRNA (red) or control siRNA (blue; n = 3). EDI inclusion levels of methylated relative to unmethylated cells were determined.

All graphs show mean values \pm SEM. Student's t test was used to compare the indicated samples. See also Figures S4 and S5.

association of HP1 with splicing factors could mean that HP1's regulation of alternative splicing involves the recruitment of splicing factors to methylated DNA.

Thus far, we found that HP1 α and HP1 β , but not HP1 γ , regulate EDI alternative splicing in a methylation-dependent manner (Figure 4A) and directly bind to splicing factors (Figure 4C). To further study the underlying mechanism, we tested whether HP1 functions as an adaptor protein that recognizes H3K9me3 and recruits splicing factors to the EDI alternative exons. We overexpressed the HP1-bound splicing factors SF3B1, SRSF1, and SRSF3 in cells in which EDI was either methylated or unmethylated. Overexpression of SRSF1 or SF3B1 did not change the basic positive shift in EDI inclusion that accompanies methylation and is observed in the GFP control, whereas overexpression of SRSF3 reduced the positive splicing shift caused by DNA methylation (Figure 4D); this same result was observed previously when overexpressing HP1 α or HP1 β (Figure 4A), which suggests that HP1 might be responsible for recruiting SRSF3 to the methylated EDI gene. SRSF3's role as a splicing silencer in the EDI system was previously described (de la Mata and Kornblihtt, 2006). Therefore, overexpressing SRSF3 is expected to lower EDI alternative exon inclusion levels regardless of the methylation state. A deeper examination of the data shows that this is indeed the case; SRSF3 overexpression decreases EDI inclusion in both methylated and unmethylated states, but the magnitude of the effect differs significantly. After overexpressing SRSF3, inclusion of the EDI alternative exons shows a 6-fold drop when the gene is methylated but only a 3-fold drop when the gene is unmethylated (Figure S5A; Student's t test, p value = 0.0034). The stronger effect of SRSF3 on a methylated EDI can be explained by additional recruitment of SRSF3 through HP1 only in a methylated state. To confirm this hypothesis, we overexpressed SRSF3 while simultaneously knocking down HP1 α and HP1 β . Indeed, in the absence of HP1 α and HP1 β , overexpression of SRSF3 produced a significantly diminished effect on EDI splicing (Figure 4E). The diminished SRSF3 effect was limited only to the methylated EDI (Figures S5B and S5C) and was not a result of differences in SRSF3 transfection levels (Figure S5D). We also performed the reciprocal experiment, where we overexpressed HP1 α and HP1 β while simultaneously knocking down SRSF3. The results further strengthen this hypothesis, as in the absence of SRSF3, overexpression of HP1 produced a significantly diminished effect on EDI splicing (Figure 4F), which suggests that SRSF3 is required for HP1's methylation-dependent effect on splicing. Put together, these findings strongly support a methylation-dependent effect of SRSF3 on the EDI alternative exons that is mediated by HP1 α and HP1 β .

Overall, we present here a mechanism that encompasses both DNA and RNA levels and is controlled epigenetically: methylated EDI alternative exons are enriched with the H3K9me3 modification, which acts as the substrate for HP1 proteins. HP1 proteins are therefore found particularly on the methylated EDI alternative exons. The strong association of HP1 α and HP1 β with SRSF3 enhances SRSF3's role as a splicing silencer in this system, lowering the inclusion levels of the EDI alternative exons, and creates the final link between DNA methylation, HP1, and splicing regulation.

DISCUSSION

The question regarding the impact of DNA methylation on the splicing process has been raised numerous times in recent years, yet knowledge about DNA methylation's regulatory effect remains limited and incomplete. Here, we use a genome-wide approach to discern DNA methylation's global ability to regulate alternative splicing. The various deep-sequencing experiments that we performed on methylation-deficient cells highlight the significant global impact that DNA methylation has on alternative splicing, both positive and negative. Altered DNA methylation levels affect the splicing of more than one-fifth of alternative exons, showing a surprisingly extensive range of influence. In contrast, DNA methylation did not have a profound effect on constitutively spliced exons, as almost all constitutive exons in wild-type cells remain so in TKO cells. We therefore suspect that DNA methylation is more of a "fine-tuning" mechanism and is unable to overcome the strong splicing signals inherent in constitutive exons. Taking into account DNA methylation's significant influence on splicing of alternative exons, this epigenetic modification will have a major contribution to current splicing prediction tools such as the "splicing code" (Barash et al., 2010), and we strongly support its introduction to these tools.

The finding that constitutive exons exhibit higher methylation levels than alternative exons implies that DNA methylation promotes exon inclusion. However, we propose that DNA methylation's fine-tuned regulation of splicing is only made apparent in alternative exons whose recognition, unlike constitutive exons, is not strongly controlled by the basic splicing recognition factors. When analyzing alternative exons that are affected or unaffected by methylation, we find that the underlying mechanism is indeed more complex: high methylation levels will repress recognition of alternative exons, while low methylation levels will enhance their recognition. We hypothesize that constitutive exons might have high inclusion in spite of and not because of high DNA methylation levels, as these exons are controlled by much stronger intrinsic factors that overshadow DNA methylation's weaker effects.

The search for a mediator protein that can affect splicing regulation on the one hand and is selective to DNA methylation on the other led us to examine the HP1 protein family. Our global analysis of the HP1 proteins points to significant similarities between HP1 and DNA methylation regarding their effect on alternative splicing. Quite strikingly, our combined genome-wide results demonstrate that for 152 alternative exons, which represent 20% of the overall effect of DNA methylation on splicing, splicing regulation can be explained by an interaction with HP1. This correlation is highly significant compared to random chance. The number of exons that are affected here is not large, but it reflects other mechanisms of refined splicing regulation by DNA binding proteins such as those published by a landmark study done by Luco et al. showing that 65 exons, which correspond to 14% of PTB-regulated exons, could be explained by regulation through MRG15 (Luco et al., 2010). In addition to DNA methylation, there might be other potential mechanisms for HP1-mediated alternative splicing, as HP1 was previously shown to bind to RNA (Muchardt et al., 2002). The existence of other, methylation-independent mechanisms could explain the non-overlapping

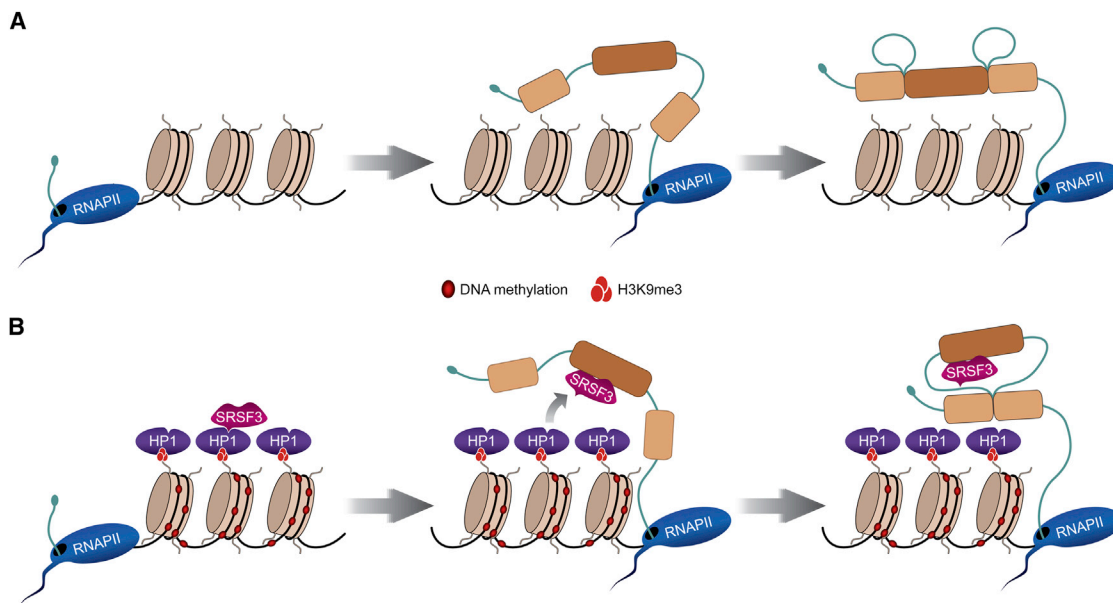


Figure 5. A Model for How HP1 Links DNA Methylation to Alternative Splicing in the EDI System

(A) When DNA is unmethylated, RNAPII (blue) transcribes the pre-mRNA molecule and the EDI alternative exon (dark brown) is included in the mature transcript. (B) When DNA is methylated (dark red circle), the histone modification H3K9me3 (three red circles) is present and HP1 (purple) binds to the chromatin. The splicing factor SRSF3 (pink) binds to HP1 and is transferred to the pre-mRNA, resulting in skipping of the EDI alternative exon in the mature transcript.

alternative splicing events. Overall, our results suggest that HP1 serves an important function as a mediator between DNA methylation and splicing, thus adding another layer of regulation to other known mechanisms of splicing regulation through DNA methylation such as CTCF (Shukla et al., 2011) and MeCP2 (Maunakea et al., 2013). Remarkably, our ChIP-seq results demonstrate a localization specific effect for HP1 on RNA splicing (Figure 2F): HP1 enhances the inclusion of exons when bound immediately upstream to the exon and silences exon recognition when bound to the exon itself. This position-specific effect has been observed before for multiple RNA binding proteins such as PTB (Lorian et al., 2010) and hnRNP proteins (Huelga et al., 2012) and is shown here for the first time for a DNA binding protein.

A strong backup to our genome-wide analysis was performed using a newly introduced system that uses site-specific targeting of a methylated gene versus an unmethylated gene. This system produced two major discoveries.

First, the overwhelming majority of papers dealing with regulation of alternative splicing by DNA methylation make use of correlational observations. Consequently, the major question in the field—whether there is a direct link between methylation and splicing—remained unanswered. Here, using our EDI experimental system, we were able to switch on or switch off DNA methylation in a single gene while keeping the endogenous background unchanged. This enormous advantage allowed us to overcome that common limitation and demonstrate for the first time a causal relationship between DNA methylation and alternative splicing.

Second, using our EDI experimental system, we show that HP1 is an adaptor protein that connects DNA methylation to

splicing by recruiting splicing factors. We found that HP1 α and HP1 β bind to methylated EDI alternative exons and recruit the splicing factor SRSF3, thus enhancing SRSF3's role as a splicing silencer in this system and lowering the inclusion levels of the EDI alternative exons in their methylated state. DNA methylation has been shown previously to regulate splicing through an impact on the kinetics of RNAPII elongation (Maunakea et al., 2013). However, the mechanism presented here represents the first reported example of DNA methylation's ability to regulate alternative splicing through the recruitment model. We therefore propose a model by which HP1 binds to the DNA methylation-associated histone modification H3K9me3 and recruits splicing factors specifically to methylated alternative exons (Figure 5). This recruitment reduces exon recognition in the EDI system, where HP1 binds to the EDI alternative exons. HP1 can also enhance exon recognition in other cases, where it binds upstream to the alternative exons, as our results show a localization-specific effect for HP1 on splicing (Figure 2F).

Our RNA-seq experiments show that all three HP1 isoforms can regulate alternative splicing. Moreover, our results display a high level of overlap (70%–80%) between the exon populations that were affected by the different HP1 proteins (Figure S2), suggesting that the HP1 isoforms probably share similar modes of action. However, while this overlap is high, it is not complete, leaving the possibility for specific cases where HP1 proteins only partially overlap. The EDI system represents such a case, as both HP1 α and HP1 β affect alternative splicing similarly, whereas HP1 γ does not strongly affect alternative splicing in this system.

The involvement of HP1 in the regulation of splicing by DNA methylation may be especially relevant in pathological

conditions, such as cancer, where widespread changes in levels of DNA methylation and aberrant alternative splicing patterns have been reported (David and Manley, 2010; Hodges et al., 2009), while overexpression of HP1 has also been detected in multiple cancer cell lines and is thought to play a role in tumorigenesis (Takanashi et al., 2009). Indeed, we found several known cases where alternative exons similarly regulated by both DNA methylation and HP1 are involved in cancer: the *Mnk* splicing isoform that includes exon 13 was found to be tumor suppressive and is downregulated in breast, lung, and colon tumors (Maimon et al., 2014); exon 12 of the *Irak1* gene affects its ability to phosphorylate and activate the NF- κ B pathway (Carpenter et al., 2014) and is an oncotarget in both myelodysplastic syndromes and acute myeloid leukemia cancers (Beverly and Starczynowski, 2014; Rhyasen et al., 2013); and exon 6 of the *Myl6* gene and exon 8 of the *App* gene were found to have differential splicing patterns in breast cancer and lung cancer, respectively (Li et al., 2006; Misquitta-Ali et al., 2011). These examples highlight the importance of HP1-mediated regulation of splicing by DNA methylation on cancer progression.

Finally, it is likely that additional methylation-dependent splicing regulatory proteins exist, and the novel model system that we present here offers the scientific community a unique opportunity to probe and discover such proteins in the future.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures can be found in [Supplemental Experimental Procedures](#).

Cells and Plasmids

Flp-In-HEK293 cells were from Invitrogen. Mouse R1 ESCs were from ATCC. Dnmt TKO ESCs were a kind gift from Prof. Masaki Okano (RIKEN) and have been previously described previously (Tsumura et al., 2006). The expression plasmids encoding HP1 α -GFP, HP1 β -GFP, HP1 γ -GFP, SRSF1-T7, and SRSF3-T7 were previously described (Cáceres et al., 1997; Cheutin et al., 2003). The expression plasmid encoding the SF3B1-GFP fusion protein was a kind gift from Prof. Juan Valcarcel (Centre de Regulació Genòmica). pSVEDA/FN containing the EDI minigene was a kind gift from Prof. Alberto Kornblihtt (Universidad de Buenos Aires) and has been previously described (Cramer et al., 1999). The EDI minigene was introduced into pcDNA5/FRT/TO (Invitrogen) for stable integration into the cells.

EDI Bisulfite Sequencing

DNA was extracted from cells, bisulfite converted, and PCR amplified. The PCR product was cloned into a plasmid and transformed into bacteria. DNA from several colonies was extracted and sequenced using the forward primer from the PCR reaction.

Analysis of Splicing Patterns by RT-PCR and qRT-PCR

Total RNA was reverse transcribed using SuperScript III (Invitrogen). For RT-PCR analysis, PCR was performed using Biotools DNA Polymerase, and resulting products were visualized on agarose gels. For qRT-PCR, qPCR was performed using KAPA SYBR FAST on a Stratagene Mx3005P thermocycler using exon-exon junction primer pairs designed to detect the inclusion or skipping isoforms.

Overexpression and siRNA Knockdown

Expression plasmids were transfected using TransIT-LT1 (Mirus), and cells were incubated for 48 hr before RNA and proteins were extracted. In all experiments, an empty pEGFP-C3 plasmid was used for control. Knockdowns were performed using siGENOME small interfering RNAs (siRNAs) (Dharmacon). Stable cells were transfected using Lipofectamine RNAiMAX (Invitrogen) and

incubated for 96 hr before RNA and proteins were extracted. R1 and TKO cells were transfected using Lipofectamine 2000 (Invitrogen) and incubated for 48 hr before RNA and proteins were extracted.

Co-immunoprecipitation

Cells were washed with PBS, and nuclei were purified and treated with MNase to release chromatin-bound proteins, then re-suspended in immunoprecipitation (IP) buffer and incubated with antibody conjugated to protein A Dynabeads (Invitrogen). Samples were incubated with RNase A and washed with IP buffer. Proteins were eluted and subjected to western blot analysis.

ChIP

Cells were cross-linked using formaldehyde, lysed, and sonicated to obtain an average DNA size of 150–350 bp. Chromatin was incubated with antibody conjugated to protein A Dynabeads (Invitrogen). Samples were stringently washed using several buffers. DNA was eluted, treated with RNase A and Proteinase K, and incubated at 65°C to reverse the cross-links. DNA was purified using MinElute PCR purification kit (QIAGEN) followed by qPCR analysis.

Library Preparation and Deep Sequencing

RNA-seq library preparation was performed using commercially available kits from Illumina. Deep sequencing was carried out on an Illumina Genome Analyzer II or Illumina HiSeq 2000.

ACCESSION NUMBERS

The GEO accession number for the sequence data reported in this paper is GSE64910.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.01.038>.

AUTHOR CONTRIBUTIONS

A.Y. designed and carried out all experiments following consultations with G.A. and E.M.; R.S. performed EDI bisulfite sequencing and HP1 overexpressions and knockdowns and helped with the creation of stable cells; S.M. maintained the mouse embryonic stem cells; S.G. performed all computational analyses with help from O.G.; A.Y. and S.G. wrote the manuscript; and A.Y., S.G., R.S., G.A., and E.M. discussed the results and commented on the manuscript.

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